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Themed Section: Molecular Pharmacology of G Protein-Coupled Receptors

REVIEW ARTICLE

Superagonism at G protein-coupled receptors and beyond

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Ligands targeting GPCRs can be categorized according to their intrinsic efficacy to trigger a specific, receptor-mediated response. A ligand endowed with the same level of efficacy as the endogenous agonist can be classified as a full agonist, whereas a compound that displays greater efficacy, that is, higher receptor signalling output than the endogenous agonist, can be called a superagonist. Subsequent to GPCR activation, an intracellular signalling cascade is set in motion, which may generate substantial amplification of the signal. This may obscure superagonism in pharmacological assays and, therefore, the definition of superagonism necessitates a combination of operational approaches, reduction of spare receptors or estimation of receptor activation close to the receptor level to quantify relative agonist efficacies in a particular system.

The first part of this review will compare GPCR superagonism with superagonism in the field of immunology, where this term is well established. In the second part, known GPCR superagonists will be reviewed. Then, the experimental and analytical challenges in the deconvolution of GPCR superagonism will be addressed. Finally, the potential benefit of superagonism is discussed.

The molecular mechanisms behind GPCR superagonism are not completely understood. However, crystallography shows that agonist binding alone is not sufficient for a fully active receptor state and that binding of the G protein is at least equally important. Accordingly, the emerging number of reported superagonists implies that ligand-induced receptor conformations more active than the ones stabilized by the endogenous agonist are indeed feasible. Superagonists may have therapeutic potential when receptor function is impaired or to induce negative feedback mechanisms.

LINKED ARTICLES

This article is part of a themed section on Molecular Pharmacology of G Protein-Coupled Receptors. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v173.20/issuetoc

Abbreviations

BRET, bioluminescence resonance energy transfer; DMR, dynamic mass redistribution; E_{max}, maximum-inducible response, asymptote of the concentration-effect curve; Oxo M, Oxotremorine M; TRH, thyrotropin-releasing hormone; [^{35}S]GTPγS, guanosine 5′-O-(γ –thio)triphosphate

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These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a b c]Alexander *et al.,* 2013a, b, c).

Introduction

G protein-coupled receptors (GPCRs) are cell surface receptors, crucial for signal transduction across cell membranes (Millar and Newton, 2010) and represent one of the largest and most diverse protein families in the human genome (Oldham and Hamm, 2008; Alexander et al., 2013a). In humans, GPCRs are encoded by about 800 different genes (Fredriksson *et al.*, 2003; Bjarnadóttir *et al.*, 2006) and mediate most cellular responses to hormones, neurotransmitters and other sensory inputs like vision, olfaction and taste (Rosenbaum *et al.*, 2009). Based on phylogenetic criteria, the large superfamily of human GPCRs can be subdivided into the five main subfamilies Glutamate, Rhodopsin, Adhesion, Frizzled/Taste and Secretin ('GRAFS' nomenclature) (Fredriksson *et al.*, 2003), among which the Rhodopsin family (resembling the class A GPCR family in the Kolakowski/NC-IUPHAR extended nomenclature system (Kolakowski, 1994; Foord *et al.*, 2005)) is by far the largest and most studied subclass. So far, more than one-third of all drugs target GPCRs (Overington *et al.*, 2006), and an emerging number of still 'undrugged' receptors display association with various diseases (e.g. Garland, 2013). This implies that GPCRs may become even more important drug targets in the future and explains why so much effort is still being put into GPCR drug discovery.

The canonical view of GPCR signal transduction is focused on the activation of intracellular heterotrimeric guanine nucleotide binding proteins (G proteins) (Milligan and Kostenis, 2006; Dohlman, 2015). In addition, G protein-independent signalling pathways are well established (Pierce and Lefkowitz, 2001; Pierce *et al.*, 2002). The ability of a ligand to elicit a receptor-mediated physiological or pharmacological response is addressed by the term 'efficacy' (Stephenson, 1956; Kenakin, 1995a; Kenakin, 2002; Kenakin, 2013; Rajagopal, 2013), and ligands can be categorized accordingly (Kenakin, 1995a; Smith *et al.*, 2011). Historically, the efficacy of a specific ligand is derived from concentration-effect curves and quantified by the maximum effect (E_{max}) relative to E_{max} of a standard compound such as the endogenous agonist (Strange, 2008; Smith *et al.*, 2011; Langmead and Christopoulos, 2013). Test systems include isolated organs, GPCR-linked second messenger accumulation and regulation of gene expression. In this regard, it is important to note that E_{max} parameters are assay-dependent and system-dependent (Langmead and Christopoulos, 2013). Drugs that induce the maximum response of a system may nevertheless differ in efficacy, because the fraction of receptors required to be agonist-bound may well differ between different agonists. This fraction depends on the individual efficacy of a given agonist for receptor activation (Nickerson, 1956; Stephenson, 1956; Kenakin, 1985). Notably, in assays, which monitor signalling outcome distal from receptor activation, the signalling response that can be measured has usually a certain assay-dependent limit, and the signal is often substantially amplified (Kenakin, 2002; Milligan, 2003; Colabufo *et al.*, 2007). As a consequence, the assay will fail to discriminate between agonists that are endowed with high, yet differing, efficacy. Approaches such as the operational model of agonism (Black and Leff, 1983) and partial receptor inactivation (e.g. Furchgott, 1966; Furchgott and Bursztyn, 1967) are therefore necessary to quantify relative estimates of ligand efficacy (e.g. Christopoulos and El-Fakahany, 1999).

Classification of GPCR ligands in the light of the term 'superagonism'

Ligands that produce the full biological response in a particular system can be designated full agonists, whereas ligands

that produce only a submaximal response even at receptor saturating concentrations are classified as partial agonists (Kenakin, 1985; Rosenbaum *et al.*, 2009; Nygaard *et al.*, 2013). This classification may necessitate ligand re-classification each time a new ligand with a higher systems response is identified. Additionally, a drug can elicit a full systems response without having full intrinsic efficacy. Therefore, as used in this article, ligands can be also classified according to their efficacy relative to the endogenous agonist: ligands, which have the same intrinsic efficacy for receptor activation as the endogenous agonist in a specific system, can be defined as full agonists. Consequently, compounds, which do not activate the receptor to its full extent even at receptor-saturating concentrations, are referred to as partial agonists, whereas ligands that display higher efficacy than the endogenous agonist are classed as "superagonists" (Figure 1A) (Smith *et al.*, 2011). Major difficulties in estimating efficacy result from high receptor densities on overexpressing cells and a certain degree of signalling amplification in pharmacological assays. This may result in a high receptor reserve, that is, only a small fraction of receptor available needs to be occupied to evoke a maximum systems response (Nickerson, 1956; Stephenson, 1956; Ariens *et al.*, 1960). Comparison of maximum effects will show that in a system with high receptor density, two agonists with different levels of intrinsic efficacy may both appear as full agonists, whereas in a biological system with low receptor density, one agonist may act as a partial agonist compared with the other (Figure 1B) (e.g. Rajagopal *et al.*, 2011; Langmead and Christopoulos, 2013; Shonberg *et al.*, 2014). As mentioned earlier, this may necessitate application of inactivation methods and functional data analysis with operational approaches to estimate relative ligand efficacies (Christopoulos and El-Fakahany, 1999).

Notably, the term efficacy always has a certain 'quality', as ligands with the ability to activate the same receptor protein may stabilize different active receptor conformations, which in turn activate different subsets of intracellular adaptor proteins leading to diverse functional responses (Kenakin, 1995b; Deupi and Kobilka, 2010; Kenakin and Christopoulos, 2013). This 'biased agonism' or 'functional selectivity' has become increasingly interesting for potential therapeutic exploitation in drug discovery (Shonberg *et al.*, 2014).

This article aims to review GPCR agonists, which are able to activate the receptor to an even higher degree than the endogenous agonist does, that is, 'superagonists' (Engström *et al.*, 2005; Smith *et al.*, 2011; Schrage *et al.*, 2013), and to highlight the potential exploitation of superagonists for both basic GPCR research and therapeutic use. Of note, application of the term 'superagonist' in biomedical data bases and search engines reveals that this designation is most commonly used in immunological research where cell signalling is mainly mediated by kinase-associated receptors or receptor kinases. In the following section, we will first focus on the term superagonism in immunology regarding similarities and differences compared with GPCR superagonism. We will then report on examples of GPCR superagonism and difficulties in the identification of compounds exceeding the endogenous agonist in efficacy. We would point out that there are also several examples of compounds with supraphysiological efficacy at ligand-gated ion channels (Carlier *et al.*, 2002; Thompson and Lummis, 2013).

Consequently, we propose that superagonism is evident not only at GPCRs but may also be applicable to all main receptor classes, which mediate physiological actions in response to endogenous agonists.

Up until now, the term 'superagonist' has not been addressed in the NC-IUPHAR nomenclature system (Neubig *et al.*, 2003). In the literature, the term is also used for agonists that are endowed with higher binding affinity compared with the endogenous activator. For example, 'superagonists' of the gonadotropin-releasing hormone receptor, such as the synthetic nonapeptide buserelin, have usually higher biological potency but not necessarily higher efficacy than the endogenous agonist gonadotropin-releasing hormone (Loumaye *et al.*, 1982; Padula, 2005; Leaños-Miranda *et al.*, 2006). However, more-than-physiological receptor binding does not necessarily accompany more-than-physiological efficacy for receptor activation.

Superagonism in immunology

The immune system is crucial for fighting off acute infections and mediating long-term protection from various pathogens

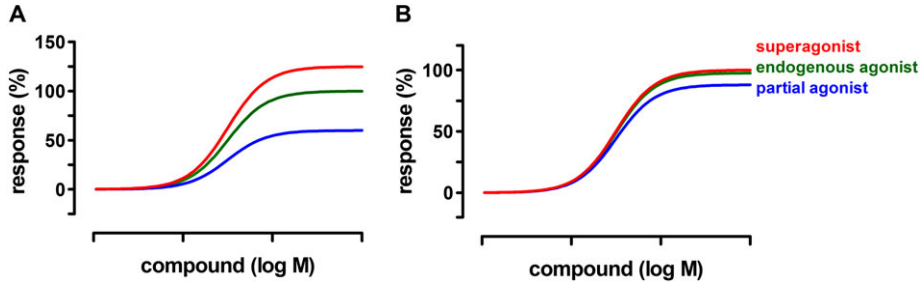


Figure 1

Signal amplification may obscure superagonism. 0% is defined by the basal level of the effect. 100% is the maximum effect of the endogenous agonist, classified as 'full agonist'. Concentration-response relationships were simulated by plotting the four-parameter logistic function (Barlow and Blake, 1989) for a set of receptor ligands differing in capacity for receptor activation using GraphPad Prism 5. (A) In a system with no or weak signal amplification: the maximum effect of the superagonist exceeds the maximum effect of the physiological agonist (taken to define the 100% level). Superagonism is obvious. (B) In a system with powerful signal amplification and in which the endogenous full agonist is taken to define the 100% level of the effect, the superagonism is masked. Note: direct experimental identification of superagonism requires use of a sensitive test system.

and malignancies. However, if it turns against endogenous or innocuous antigens, it can also cause harm. T cells play a central role in both protective and deleterious immune responses. They can be subdivided in pro-inflammatory (effector T cells (T_{eff}s)) or suppressive T cells (regulatory T cells (T_{reg}s)). In this regard, compounds, which hyperstimulate either immune-inhibitory T_{reg}s or pro-inflammatory T_{eff}s, may be of great therapeutic value for the treatment of autoimmune disorders or anti-cancer therapy respectively. There are several examples for immunological 'superagonists' that, in comparison with the endogenous ligands, display enhanced biological activity for triggering immune cell activation and proliferation (Chen *et al.*, 2000; Beyersdorf *et al.*, 2005; Rubinstein *et al.*, 2006; Abdul-Alim *et al.*, 2010).

The best established example of immunological superagonists are *anti-inflammatory* CD28-binding antibodies, which were discovered in 1997 (Tacke *et al.*, 1997). In contrast to the natural CD28-ligands, CD80 or CD86, CD28-binding antibodies can fully activate anti-inflammatory T_{reg}s without the need of T cell receptor activation, which is usually required (Lenschow *et al.*, 1996; Tacke *et al.*, 1997; Janeway *et al.*, 2001) (Figure 2A). Binding of the endogenous ligands to CD28 does not elicit a biological response (Figure 2B), suggesting that similar to GPCR superagonism, the conformation of CD28 stabilized by superagonist CD28 antibodies is distinct from that stabilized by CD80 or CD86.

Examples of immunological *pro-inflammatory* superagonists are highly active cytokines complexed to a soluble subunit of their receptor (Fischer *et al.*, 1997; Pflanz *et al.*, 1999; Rubinstein *et al.*, 2006), which trigger increased activation of target cells, such as T cells and natural killer cells, and altered peptide ligands stimulating activation of cytotoxic T cells to a greater extent than the natural peptide (Bakker *et al.*, 1997; Valmori *et al.*, 1998; Chen *et al.*, 2000).

Taken together, distinct classes of immunological superagonists act through diverse mechanisms resulting in a more-than-physiological ('superagonist') biological activity, that is, enhanced cell growth and proliferation of a subset of immune cells. There are obvious similarities to 'GPCR superagonism', which is defined as receptor activation with a superior efficacy in comparison with that of the endogenous agonist (Smith *et al.*, 2011). However, biological activity of immune-stimulating compounds is assessed by cell growth and proliferation in a multi-layered interplay between different immunological cell types. Under these complex conditions, compounds with supraphysiological efficacy for activation of a certain receptor are difficult to identify. This is because greater biological activity might be due to an increased affinity for receptor binding or functional selectivity for a certain signalling pathway and, therefore, is not necessarily due to higher efficacy for receptor activation on the receptor protein level.

Class A GPCR superagonists

As discussed earlier, ligands that target GPCRs are usually classified according to their efficacy, that is, their ability to elicit a receptor-mediated physiological or pharmacological response (Stephenson, 1956; Kenakin, 1995a; Smith *et al.*, 2011). By intuition, the interaction of the endogenous transmitter with its cognate receptor might be assumed as efficacious as possible because it is the consequence of a strong evolutionary force (Langmead and Christopoulos, 2013). This would preclude a more-than-physiological ligand efficacy. However, crystallographic efforts with GPCRs in their active state show that agonist binding alone is not sufficient to stabilize a fully active

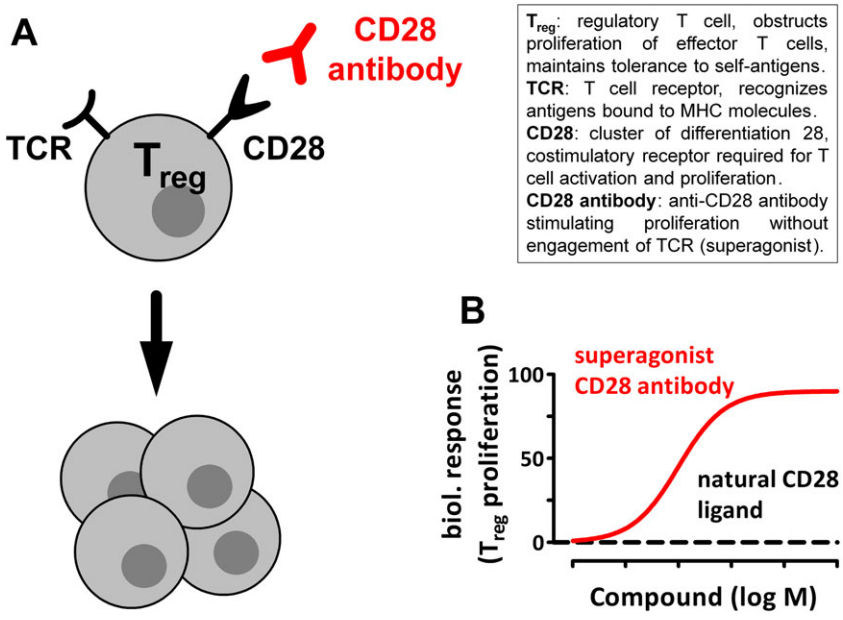


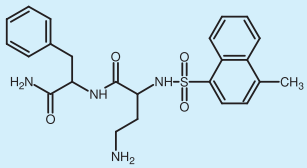
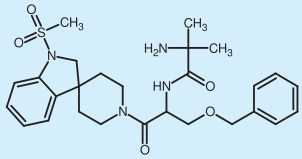
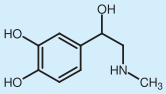
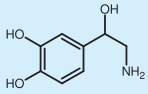
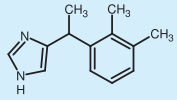
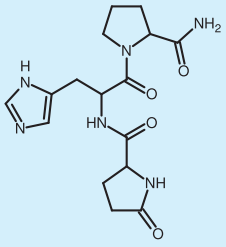
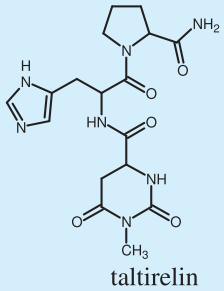
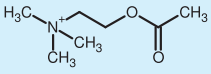
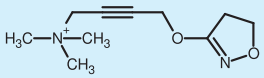
Figure 2

Superagonists of CD28 induce activation and proliferation of regulatory T cells. (A) Antibodies targeting the T cell surface receptor CD28 can induce activation and proliferation of regulatory T cells (T_{reg}s) without the need of T cell receptor activation. (B) Hypothetical concentration-response curves illustrating CD28-mediated induction of T_{reg} proliferation by the natural CD28 ligands CD80 or CD86 (black dashed line) or a superagonistic CD28 antibody (red).

receptor conformation (Rosenbaum *et al.*, 2011) and that binding of a G protein or a G protein-mimicking nanobody is at least as important to capture the protein in a fully active state (Rasmussen *et al.*, 2011b; Rasmussen *et al.*, 2011a). Moreover, recent studies show that GPCRs exist in ensembles of conformations and that agonists stabilize only a subset of possible conformational states (Deupi and Kobilka, 2007; Kobilka and Deupi, 2007; Kenakin, 2013). Therefore, diverse agonists of a given receptor protein may stabilize different subsets of conformations with distinct efficacies for the activation of specific signalling pathways (Kenakin, 2013). This is also supported by NMR spectroscopy studies showing that even 'strong' agonists alone populate a *set* of conformations similar to the more uniform and fully active conformation generated by a highly efficacious agonist plus a G protein mimicking nanobody (Nygaard *et al.*, 2013). Consequently, at least from a theoretical point of view, supraphysiological efficacy of compounds stabilizing a more uniform conformation than the endogenous agonist should in principle be feasible. Indeed, in the largest and most 'druggable' class of GPCRs (the rhodopsin-like class or class A (Fredriksson *et al.*, 2003; Lagerström and Schiöth, 2008)), a few synthetic compounds have been described that are endowed with greater intrinsic efficacy than the endogenous ligand (Table 1).

Table 1

Examples of class A (Rhodopsin-like) GPCR superagonists

Endogenous ligand	'Superagonist'	GPCR subtype	Experimental evidence for superagonism
SRIF-14: Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys SRIF-28: Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	 J-2156	Somatostatin sst_4 receptor	J-2156 is a superagonist at the human sst_4 receptor as shown by [^{35}S]GTPγS binding assays, which revealed J-2156 to generate E_{max} values that were two–three times larger than the E_{max} values of the two endogenous peptides SRIF-14 and SRIF-28 (Engström *et al.*, 2005).
Ghrelin: Gly-Ser-Ser (n-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg	 ibutamoren (MK-677)	Ghrelin receptor	Ibutamoren (MK-677) is a superagonist at the ghrelin receptor as it displayed higher E_{max} values for β-arrestin activation (discovered in BRET assays) (Holst *et al.*, 2005), for SRE-mediated transcription assays (Holst *et al.*, 2005), and for the activation of $G\alpha_{o1}$ (Bennett *et al.*, 2009) in [^{35}S]GTPγS assays.
 epinephrine,  norepinephrine  dexmedetomidine		α_{2A}-adrenoceptor	Dexmedetomidine induced higher E_{max} values for α_{2A} adrenoceptor-mediated MAP kinase activation than adrenaline (Tan *et al.*, 2002).
 TRH	 taltirelin	Thyrotropin-releasing hormone TRH_1 receptor	Taltirelin is a superagonist at the human TRH_1 receptor because it increased cellular IP_1 to E_{max} = 180% of that induced by TRH (Thirunarayanan *et al.*, 2012).
 ACh	 iperoxo	Muscarinic M_2 cholinoceptor	Iperoxo is a superagonist at muscarinic M_2 receptors for $G\alpha_i$ and $G\alpha_s$ –mediated DMR as it displayed higher operational efficacy (τ) (Schrage *et al.*, 2013). Iperoxo induced more pronounced intracellular loop rearrangement than the endogenous agonist ACh (Bock *et al.*, 2012).

SRIF-14, SRIF-28 are the endogenous forms of somatostatin and ligands for the somatostatin receptor

One example is the somatostatin receptor superagonist J-2156 [(1′S,2S)-4-amino-N-(1′-carbamoyl-2′-phenylethyl)-2-(4″-methyl-1″-naphthalenesulfonylamino)butanamide], which binds to the human somatostatin sst_4 receptor with subnanomolar affinity (Engström *et al.*, 2005). J-2156 induced sst_4 receptor-mediated [^{35}S]GTPγS binding in CHO membranes with a signal two–three times as large as the response induced by the two endogenous peptides somatostatin SRIF-28 and SRIF-14. In contrast, maximal inhibition of intracellular cAMP levels, a cellular signalling event that occurs downstream of GPCR/G protein activation, was comparable between J-2156 and the two natural peptides (Engström *et al.*, 2005). This example illustrates that the assessment of ligand efficacy distal from receptor activation may be difficult because the signal can be substantially amplified inside the cell (e.g. Milligan, 2003; Colabufo *et al.*, 2007). Such signal amplification after activation of a G_s-coupled receptor is illustrated in Figure 3. One active receptor protein may lead to the activation of several intracellular kinases, which finally shape the cellular response. Therefore, quantification of agonist efficacy by E_{max} values (i.e. the asymptote of the concentration-effect curve) is likely to obscure superagonism in an assay system characterized by strong signal amplification.

The ghrelin receptor activates a plethora of signalling pathways, and several compounds that can engage one or the other pathway with supraphysiological efficacy have been described (Holst *et al.*, 2005; Bennett *et al.*, 2009). One of these compounds is ibutamoren (MK-677), which acts as full agonist regarding Ca^{++} mobilization and inositol phosphate accumulation but displayed superagonism in 'serum-responsive element' (SRE)-mediated transcription assays (Holst *et al.*, 2005), β-arrestin recruitment (Holst *et al.*, 2005) and $G\alpha_{o1}$ activation (Bennett *et al.*, 2009). This example shows that superagonism does not necessarily occur in all possible signalling pathways that can be engaged by a certain receptor protein. This is also the case for dexmedetomidine, an α_{2A}-adrenoceptor agonist, which had higher E_{max} value for MAP kinase activation than adrenaline but was a partial agonist in [^{35}S]GTPγS binding assays performed with HEK293-α_{2A} membranes (Tan *et al.*, 2002).

In a study performed by Thirunarayanan *et al.*, the pharmacology of taltirelin, the only analogue of the thyrotropin-releasing hormone (TRH) approved for use in humans, was investigated (Thirunarayanan *et al.*, 2012). Taltirelin is used in Japan for the treatment of adult spinal muscular atrophy. In comparison with TRH, taltirelin had a lower affinity for the human TRH_1 receptor in whole cell binding experiments and also a lower potency, while showing the same E_{max} for the induction of Ca^{++} release. A first indicator of supraphysiological efficacy was the higher affinity/potency ratio of taltirelin compared with TRH, which has been used before to estimate efficacy and identify superagonists (Engel *et al.*, 2006). When TRH_1 receptor-induced inositol monophosphate (IP_1) accumulation, a cellular event upstream of receptor-mediated Ca^{++} release, was quantified, taltirelin stimulated an increase in IP_1 production that was 180% of that stimulated by TRH, identifying taltirelin as a superagonist. Similar to the study by Engström *et al.* (2005), the study by Thirunarayanan *et al.*

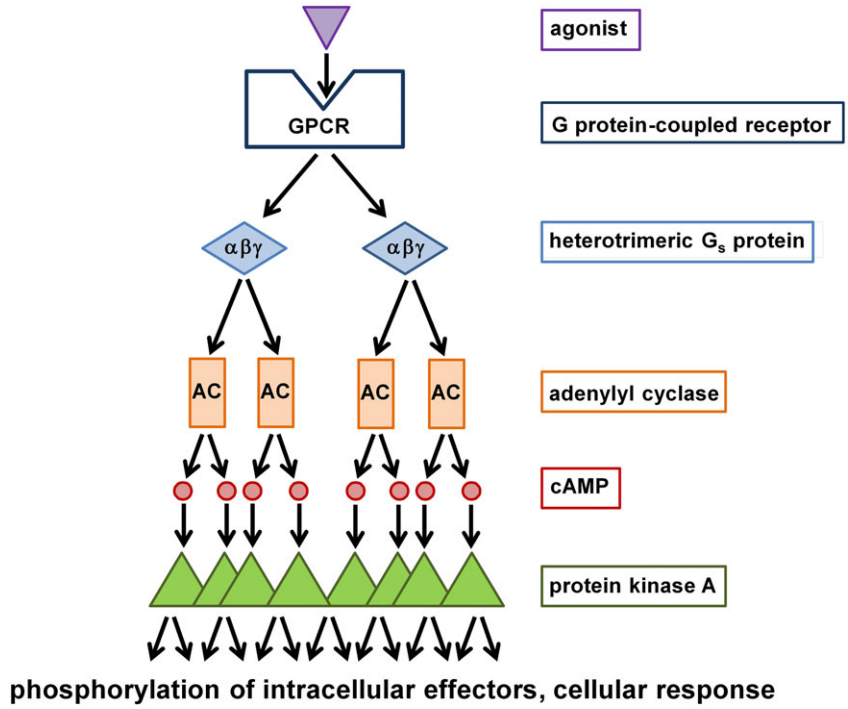


Figure 3

GPCR signalling may undergo substantial cellular amplification. As an example, the diagram shows an intracellular signalling cascade initiated by the activation of a G_s-linked GPCR. Upon activation, the receptor may activate more than one heterotrimeric G protein, which can activate more than one membrane-bound adenylyl cyclase isoforms (AC). ACs, in turn, catalyse the generation of several molecules of cAMP. cAMP binds and activates kinases such as protein kinase A, which are responsible for the phosphorylation of various intracellular effector proteins finally shaping the ultimate cellular response.

demonstrates a substance's superagonism by comparison of E_{max} values in functional assays detecting events close to the receptor. Of note, in both studies, superagonism was masked when more distal cellular events (in this case Ca^{++} release) were used to quantify receptor activation (Thirunarayanan *et al.*, 2012).

Challenges in the detection of superagonism: examples from muscarinic receptors

Similar to the receptors discussed earlier, muscarinic ACh receptors belong to the rhodopsin-like or class A GPCRs (e.g. Kruse *et al.*, 2014). Two structurally similar compounds, iperoxo (Schrage *et al.*, 2013) and oxotremorine M (Oxo M) (Mistry *et al.*, 2005), have been identified as agonists with supraphysiological efficacy at the closely related muscarinic M_2 and M_4 receptors respectively. Both receptors preferentially activate inhibitory G_i proteins (e.g. Caulfield, 1993; Wess *et al.*, 1997) but for both, M_2 (Michal *et al.*, 2001; Bock *et al.*, 2012; Schrage *et al.*, 2013) and M_4 receptors (Dittman *et al.*, 1994; Mistry *et al.*, 2005), the activation of stimulatory G_s proteins has also been reported. Of note, Oxo M and iperoxo (Table 1) are bulkier than the endogenous agonist ACh and may therefore form more interactions with the receptor, which in turn leads to higher efficacy (Schrage *et al.*, 2013).

In the study performed by Mistry *et al.* (2005), the classical muscarinic agonist Oxo M and methacholine (used as a surrogate for the endogenous agonist ACh in this study) shared the same maximum for M_2 and M_4 receptor-mediated G_i and G_s activation. The relative efficacy values (E_{rel}), according to the method of Ehlert (1985), quantified the coupling of receptor occupancy to adenylyl cyclase functional responses and revealed that, for both G_i and G_s pathways, Oxo M was a full agonist at M_2 but a superagonist at M_4 receptors, relative to methacholine. However, although highly similar, methacholine is structurally distinct from the endogenous agonist ACh, the latter not being included in this study (Mistry *et al.*, 2005). Consequently, the greater efficacy of Oxo M compared with that of ACh remains to be demonstrated.

Recently, the muscarinic agonist iperoxo, a derivative of Oxo M, which binds to muscarinic receptors with outstanding affinity (Dallanoce *et al.*, 1999; Antony *et al.*, 2009; Schrage *et al.*, 2013; Schrage *et al.*, 2014) and has been extensively exploited as a building block for muscarinic ortho-allosteric hybrid compounds (Disingrini *et al.*, 2006; Antony *et al.*, 2009; Bock *et al.*, 2012; Bock *et al.*, 2014; Matera *et al.*, 2014; Chen *et al.*, 2015), was identified as a superagonist at M_2 receptors. In the study by Schrage *et al.*, (2013), iperoxo was a highly potent agonist for the activation of M_2 receptors stably expressed in CHO cells in two assays. Whole cell label-free dynamic mass redistribution (DMR) revealed highly potent activation of G_i and G_s mediated pathways. Likewise, [^{35}S]GTPγS binding experiments performed with CHO-M_2 membranes revealed that the potency for G_i activation by iperoxo was 100-fold higher than that of ACh. However, the maximum effect of iperoxo did not differ from the effects of ACh and Oxo M. As both assays may be subject to substantial signal amplification, the operational model of agonism was employed to analyse functional data from DMR and [^{35}S]GTPγS experiments. The operational efficacy parameter τ incorporates efficacy, receptor density and coupling efficiency within a system (Black and Leff, 1983; Rajagopal *et al.*, 2011; Kenakin and Christopoulos, 2013). The value of τ was significantly greater for iperoxo than for ACh and Oxo M, both for the G_i and the G_s pathways. Therefore, iperoxo was classified as a superagonist. However, like other analytical methods, the operational model of agonism may lack a certain robustness with which the model can be fitted to the data (Langmead and Christopoulos, 2013), and this is especially true for highly efficacious agonists (Rajagopal, 2013). To improve robustness of operational efficacies, the apparent agonist equilibrium binding constants K_A, derived from whole cell radioligand binding experiments, were included in the analysis. However, binding affinity may not reflect the functional affinity within the trimeric agonist–receptor–transducer complex (Kenakin *et al.*, 2012; Kenakin and Christopoulos, 2013; Langmead and Christopoulos, 2013) and, therefore, may insert a certain 'system bias' into the estimation of operational efficacies. Nevertheless, this approach suggested that iperoxo's operational efficacy was greater than that of ACh and Oxo M. (Schrage *et al.*, 2013). In two other studies, in which functional data of iperoxo and ACh were fitted to the operational model of agonism without fixing K_A values, iperoxo and ACh showed equal operational efficacy in [^{35}S]GTPγS and cAMP accumulation assays (Bock *et al.*, 2012), but iperoxo had greater operational efficacy compared with ACh in DMR experiments (Bock *et al.*, 2014). To test further for superagonism, the M_2 receptor density was reduced in order to eliminate spare receptors, by alkylation with the irreversible antagonist phenoxybenzamine (Furchgott, 1966). Under these conditions, the maximum DMR response of ACh was more compromised by receptor alkylation than the E_{max} of iperoxo (Schrage *et al.*, 2013). However, alkylation experiments may be difficult as they do not reflect equilibrium conditions and are sensitive to the time of measurement and ground state, for example, receptor number, of the system. Nevertheless, inactivation methods (Furchgott, 1966; Furchgott and Bursztyn, 1967) are most useful to estimate agonist affinity and relative efficacy and may be applied for both partial and highly efficacious agonists (Christopoulos and El-Fakahany, 1999).

Finally, when M_2 receptor activation was measured directly at the receptor level by the quantification of intracellular loop rearrangement in Förster resonance energy transfer assays, iperoxo displayed a greater agonist-induced change of receptor conformation than ACh (Bock *et al.*, 2012). Taken together, findings from various experimental approaches suggest that iperoxo has supraphysiological efficacy at the muscarinic M_2 receptor subtype. Moreover, iperoxo provides a paradigm to demonstrate the usefulness of superagonism in experimental pharmacology: the compound served to crystallize the muscarinic M_2 receptor in its active state (Kruse *et al.*, 2013). In addition, the tritiated form of iperoxo is the first radioagonist to probe all five muscarinic receptor subtypes (Schrage *et al.*, 2014).

In summary, operational approaches to quantify agonist coupling efficiency are useful to probe for GPCR superagonism. However, operational efficacies should be considered with caution regarding data robustness. To overcome the lack of robustness, one might be tempted to reduce flexibility from the operational model by including the experimentally measured, apparent agonist equilibrium binding constant K_A. However,

this may insert system bias to the data. If researchers aim at the identification of superagonism, it is useful to apply additional routes to check for supraphysiological efficacy such as receptor alkylation experiments, estimation of receptor activation close to the receptor level or the direct probing of agonist-induced conformational transitions.

Superagonism at other receptor classes

There are several examples (Carlier *et al.*, 2002; Ihara *et al.*, 2004; Brown *et al.*, 2006; Thompson and Lummis, 2013) for supraphysiological agonist efficacy at ligand-gated ion channels. The study of ion channel activation by ion fluxes may allow a rather direct approach to measure agonism at the receptor level without complicating signal amplification. GABA is the major inhibitory transmitter in the vertebrate central nervous system and is an agonist at three different GABA receptor subtypes. In 2002, Carlier *et al.* identified a superagonist at the $GABA_A$ receptor, a ligand-gated chloride ion channel. This compound, a GABA amide dimer (compound 5b) was 33-fold less potent than GABA but induced a chloride uptake that was 49% higher than that achieved by GABA (Carlier *et al.*, 2002). Another example is the 5-HT_3 receptor at which m-chlorophenylbiguanide is a superagonist. In 5-HT_3AB receptor heteromers it produced a response 2.6-fold higher than that of 5-HT (Thompson and Lummis, 2013).

There are also reports that assign superagonism to other targets such as receptor tyrosine kinases (Puddicombe *et al.*, 1996; Thomas *et al.*, 2008) or receptors with transcriptional activity (Hourai *et al.*, 2008). However, the functional readouts for these receptors are often distal of receptor activation, that is, cell growth/proliferation and gene expression. Such 'down-stream readouts' may be subject to amplification, cross-regulation of pathways or signal convergence. Therefore, it is not surprising that many of these studies are identifying ligands with higher affinity, but not necessarily higher efficacy, than the endogenous ligand.

Conclusion

This review aims to give an overview of superagonism in different receptor classes, that is, GPCRs, catalytic receptors associated with kinases, ion channels and other target receptors, with a focus on GPCR superagonism. Superagonists may have great value as tools in experimental pharmacology or may serve to overcome loss-of-function receptor mutants, stimulate inhibitory receptors or induce negative feedback mechanisms.

Although not yet officially recognized by the NC-IUPHAR (Neubig *et al.*, 2003), there are several examples for agonists with a more-than-physiological efficacy especially at GPCRs (Tan *et al.*, 2002; Engström *et al.*, 2005; Holst *et al.*, 2005; Mistry *et al.*, 2005; Engel *et al.*, 2006; Bennett *et al.*, 2009; Schrage *et al.*, 2013). These examples show that the interaction of a receptor with its endogenous ligand was not necessarily designed by nature to be as efficacious as possible. More likely, the evolutionary forces drove receptors and endogenous ligands to a well-working machinery that is efficient but leaves substantial flexibility in biological networks to fine-tune responses. Therefore, we would predict that compounds with supraphysiological efficacy might be generated for several members of the GPCR family. Moreover, superagonists have also been identified for ligand-gated ion channels (Carlier *et al.*, 2002; Ihara *et al.*, 2004; Brown *et al.*, 2006; Thompson and Lummis, 2013). This, and the frequent identification of superagonists for catalytic, kinase-associated receptors in immunology (Chen *et al.*, 2000; Beyersdorf *et al.*, 2005; Rubinstein *et al.*, 2006; Abdul-Alim *et al.*, 2010), implies that ligands with supraphysiological efficacy can be achieved for several, if not all, receptor classes.

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Conflict of interest

None.

References

Abdul-Alim CS, Li Y, Yee C (2010). Conditional superagonist CTL ligands for the promotion of tumor-specific CTL responses. J Immunol 184: 6514–6521.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA *et al* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Ligand-Gated Ion Channels. Br J Pharmacol 170: 1582–1606.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al* (2013c). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Antony J *et al.* (2009). Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. FASEB J 23: 442–450.

Ariens EJ, van Rossum J, Koopman PC (1960). Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested on isolated organs. Arch Int Pharmacodyn Ther (Archives internationales de pharmacodynamie et de thérapie) 127: 459–478.

Bakker AB *et al.* (1997). Analogues of CTL epitopes with improved MHC class-I binding capacity elicit anti-melanoma CTL recognizing the wild-type epitope. Int J Cancer 70: 302–309.

Barlow R, Blake JF (1989). Hill coefficients and the logistic equation. Trends Pharmacol Sci 10: 440–441.

Bennett KA, Langmead CJ, Wise A, Milligan G (2009). Growth hormone secretagogues and growth hormone releasing peptides act as orthosteric super-agonists but not allosteric regulators for activation of the G protein Galpha(o1) by the ghrelin receptor. Mol Pharmacol 76: 802–811.

Beyersdorf N, Hanke T, Kerkau T, Hünig T (2005). Superagonistic anti-CD28 antibodies: potent activators of regulatory T cells for the therapy of autoimmune diseases. Ann Rheum Dis 64: iv91–iv95.

Bjarnadóttir TK, Gloriam DE, Hellstrand SH, Kristiansson H, Fredriksson R, Schiöth HB (2006). Comprehensive repertoire and

phylogenetic analysis of the G protein-coupled receptors in human and mouse. Genomics 88: 263–273.

Black JW, Leff P (1983). Operational models of pharmacological agonism. Proc R Soc Lond B Biol Sci 220: 141–162.

Bock A *et al.* (2012). The allosteric vestibule of a seven transmembrane helical receptor controls G-protein coupling. Nat Commun 3: 1044.

Bock A *et al.* (2014). Dynamic ligand binding dictates partial agonism at a G protein-coupled receptor. Nat Chem Biol 10: 18–20.

Brown LA, Ihara M, Buckingham SD, Matsuda K, Sattelle DB (2006). Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J Neurochem 99: 608–615.

Carlier PR, Chow ES, Barlow RL, Bloomquist JR (2002). Discovery of non-zwitterionic GABA(A) receptor full agonists and a superagonist. Bioorg Med Chem Lett 12: 1985–1988.

Caulfield MP (1993). Muscarinic receptors – characterization, coupling and function. Pharmacol Ther 58: 319–379.

Chen JL *et al.* (2000). Identification of NY-ESO-1 peptide analogues capable of improved stimulation of tumor-reactive CTL. J Immunol 165: 948–955.

Chen X *et al.* (2015). Rational design of partial agonists for the muscarinic m1 acetylcholine receptor. J Med Chem 58: 560–576.

Christopoulos A, El-Fakahany EE (1999). Qualitative and quantitative assessment of relative agonist efficacy. Biochem Pharmacol 58: 735–748.

Colabufo NA, Perrone GM, Contino M, Berardi F, Perrone R (2007). Receptor–drug interaction: europium employment for studying the biochemical pathway of G-protein-coupled receptor activation. Met Based Drugs 2007: 12635.

Dallanoce C *et al.* (1999). Synthesis and functional characterization of novel derivatives related to oxotremorine and oxotremorine-M. Bioorg Med Chem 7: 1539–1547.

Deupi X, Kobilka BK (2007). Activation of G protein-coupled receptors. Adv Protein Chem 74: 137–166.

Deupi X, Kobilka BK (2010). Energy landscapes as a tool to integrate GPCR structure, dynamics, and function. Physiology (Bethesda) 25: 293–303.

Disingrini T*et al.* (2006). Design, synthesis, and action of oxotremorine-related hybrid-type allosteric modulators of muscarinic acetylcholine receptors. J Med Chem 49: 366–372.

Dittman AH *et al.* (1994). A novel mechanism for coupling of M4 muscarinic acetylcholine receptors to calmodulin-sensitive adenylyl cyclases: crossover from G protein-coupled inhibition to stimulation. Biochemistry 33: 943–951.

Dohlman HG (2015). Thematic minireview series: cell biology of G protein signaling. J Biol Chem 290: 6679–6680.

Ehlert FJ (1985). The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. Mol Pharmacol 28: 410–421.

Engel S *et al.* (2006). Low affinity analogs of thyrotropin-releasing hormone are super-agonists. J Biol Chem 281: 13103–13109.

Engström M, Tomperi J, El-Darwish K, Ahman M, Savola J, Wurster S (2005). Superagonism at the human somatostatin receptor subtype 4. J Pharmacol Exp Ther 312: 332–338.

Fischer M *et al.* (1997). I. A bioactive designer cytokine for human hematopoietic progenitor cell expansion. Nat Biotechnol 15: 142–145.

Foord SM *et al.* (2005). International Union of Pharmacology. XLVI. G protein-coupled receptor list. Pharmacol Rev 57: 279–288.

Fredriksson R, Lagerström MC, Lundin L, Schiöth HB (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63: 1256–1272.

Furchgott RF (1966). The use of B-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. In: Harper NJ, Simmonds AB (eds). Advances in Drug Research, Vol. 3. Academic Press: New York, pp. 21–55.

Furchgott RF, Bursztyn P (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann NY Acad Sci 144: 882–899.

Garland SL (2013). Are GPCRs still a source of new targets? J Biomol Screen 18: 947–966.

Holst B, Brandt E, Bach A, Heding A, Schwartz TW (2005). Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling. Mol Endocrinol 19: 2400–2411.

Hourai S *et al.* (2008). Structure-based design of a superagonist ligand for the vitamin D nuclear receptor. Chem Biol 15: 383–392.

Ihara M, Matsuda K, Shimomura M, Sattelle DB, Komai K (2004). Super agonist actions of clothianidin and related compounds on the SAD beta 2 nicotinic acetylcholine receptor expressed in Xenopus laevis oocytes. Biosci Biotechnol Biochem 68: 761–763.

Janeway CA, Traver P, Walport M, Shlomchik MJ (2001). Immunobiology. The Immune System in Health and Disease. Garland Pub: New York.

Kenakin T (1985). The quantification of relative efficacy of agonists. J Pharmacol Methods (Journal of pharmacological methods) 13: 281–308.

Kenakin T (1995a). Agonist-receptor efficacy. I: mechanisms of efficacy and receptor promiscuity. Trends Pharmacol Sci 16: 188–192.

Kenakin T (1995b). Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. Trends Pharmacol Sci 16: 232–238.

Kenakin T (2002). Efficacy at G-protein-coupled receptors. Nat Rev Drug Discov 1: 103–110.

Kenakin T (2013). New concepts in pharmacological efficacy at 7TM receptors: IUPHAR review 2. B J Pharmacol 168: 554–575.

Kenakin T, Christopoulos A (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat Rev Drug Discov 12: 205–216.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, Novick S (2012). A simple method for quantifying functional selectivity and agonist bias. ACS Chem Neurosci 3: 193–203.

Kobilka BK, Deupi X (2007). Conformational complexity of G-protein-coupled receptors. Trends Pharmacol Sci 28: 397–406.

Kolakowski LF (1994). GCRDb: a G-protein-coupled receptor database. Recept Channels 2: 1–7.

Kruse AC *et al.* (2013). Activation and allosteric modulation of a muscarinic acetylcholine receptor. Nature 504: 101–106.

Kruse AC, Kobilka BK, Gautam D, Sexton PM, Christopoulos A, Wess J (2014). Muscarinic acetylcholine receptors: novel opportunities for drug development. Nat Rev Drug Discov 13: 549–560.

Lagerström MC, Schiöth HB (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov 7: 339–357.

Langmead CJ, Christopoulos A (2013). Supra-physiological efficacy at GPCRs: superstition or super agonists? B J Pharmacol 169: 353–356.

Leaños-Miranda A, Ulloa-Aguirre A, Cervini LA, Janovick JA, Rivier J, Conn PM (2006). Identification of new gonadotrophin-releasing hormone partial agonists. J Endocrinol 189: 509–517.

Lenschow DJ, Walunas TL, Bluestone JA (1996). CD28/B7 system of T cell costimulation. Annu Rev Immunol 14: 233–258.

Loumaye E, Naor Z, Catt KJ (1982). Binding affinity and biological activity of gonadotropin releasing hormone agonists in isolated pituitary cells. Endocrinology 111: 730–736.

Matera C *et al.* (2014). Bis(ammonio)alkane-type agonists of muscarinic acetylcholine receptors: synthesis, in vitro functional characterization, and in vivo evaluation of their analgesic activity. Eur J Med Chem 75: 222–232.

Michal P, Lysíková M, Tucek S (2001). Dual effects of muscarinic M(2) acetylcholine receptors on the synthesis of cyclic AMP in CHO cells: dependence on time, receptor density and receptor agonists. B J Pharmacol 132: 1217–1228.

Millar RP, Newton CL (2010). The year in G protein-coupled receptor research. Mol Endocrinol 24: 261–274.

Milligan G (2003). Principles: extending the utility of [35S]GTP gamma S binding assays. Trends Pharmacol Sci 24: 87–90.

Milligan G, Kostenis E (2006). Heterotrimeric G-proteins: a short history. B J Pharmacol 147: S46–S55.

Mistry R, Dowling MR, Challiss RA (2005). An investigation of whether agonist-selective receptor conformations occur with respect to M2 and M4 muscarinic acetylcholine receptor signalling via Gi/o and Gs proteins. B J Pharmacol 144: 566–575.

Neubig RR, Spedding M, Kenakin T, Christopoulos A (2003). International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacol Rev 55: 597–606.

Nickerson M (1956). Receptor occupancy and tissue response. Nature 178: 697–698.

Nygaard R *et al.* (2013). The dynamic process of β(2)-adrenergic receptor activation. Cell 152: 532–542.

Oldham WM, Hamm HE (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. Nat Rev Mol Cell Biol 9: 60–71.

Overington JP, Al-Lazikani B, Hopkins AL (2006). How many drug targets are there? Nat Rev Drug Discov 5: 993–996.

Padula AM (2005). GnRH analogues – agonists and antagonists. Anim Reprod Sci 88: 115–126.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.* (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl. Acids Res. 42: D1098–1106.

Pflanz S *et al.* (1999). A fusion protein of interleukin-11 and soluble interleukin-11 receptor acts as a superagonist on cells expressing gp130. FEBS Lett 450: 117–122.

Pierce KL, Lefkowitz RJ (2001). Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. Nat Rev Neurosci 2: 727–733.

Pierce KL, Premont RT, Lefkowitz RJ (2002). Seven-transmembrane receptors. Nat Rev Mol Cell Biol 3: 639–650.

Puddicombe SM, Wood L, Chamberlin SG, Davies DE (1996). The interaction of an epidermal growth factor/transforming growth factor alpha tail chimera with the human epidermal growth factor receptor reveals unexpected complexities. J Biol Chem 271: 30392–30397.

Rajagopal S *et al.* (2011). Quantifying ligand bias at seven-transmembrane receptors. Mol Pharmacol 80: 367–377.

Rajagopal S (2013). Quantifying biased agonism: understanding the links between affinity and efficacy. Nat Rev Drug Discov 12: 483.

Rasmussen SG *et al.* (2011a). Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 477: 549–555.

Rasmussen SG *et al.* (2011b). Structure of a nanobody-stabilized active state of the β(2) adrenoceptor. Nature 469: 175–180.

Rosenbaum DM *et al.* (2011). Structure and function of an irreversible agonist-β(2) adrenoceptor complex. Nature 469: 236–240.

Rosenbaum DM, Rasmussen SG, Kobilka BK (2009). The structure and function of G-protein-coupled receptors. Nature 459: 356–363.

Rubinstein MP *et al.* (2006). Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. Proc Natl Acad Sci U S A 103: 9166–9171.

Schrage R *et al.* (2013). Agonists with supraphysiological efficacy at the muscarinic M2 ACh receptor. B J Pharmacol 169: 357–370.

Schrage R *et al.* (2014). New insight into active muscarinic receptors with the novel radioagonist [^{3}H]iperoxo. Biochem Pharmacol 90: 307–319.

Shonberg J, Lopez L, Scammells PJ, Christopoulos A, Capuano B, Lane JR (2014). Biased agonism at G protein-coupled receptors: the promise and the challenges – a medicinal chemistry perspective. Med Res Rev 34: 1286–1330.

Smith NJ, Bennett KA, Milligan G (2011). When simple agonism is not enough: emerging modalities of GPCR ligands. Mol Cell Endocrinol 331: 241–247.

Stephenson RP (1956). A modification of receptor theory. Br J Pharmacol Chemother 11: 379–393.

Strange PG (2008). Agonist binding, agonist affinity and agonist efficacy at G protein-coupled receptors. B J Pharmacol 153: 1353–1363.

Tacke M, Hanke G, Hanke T, Hünig T (1997). CD28-mediated induction of proliferation in resting T cells in vitro and in vivo without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. Eur J Immunol 27: 239–247.

Tan CM, Wilson MH, MacMillan LB, Kobilka BK, Limbird LE (2002). Heterozygous alpha 2A-adrenergic receptor mice unveil unique therapeutic benefits of partial agonists. Proc Natl Acad Sci U S A 99: 12471–12476.

Thirunarayanan N, Raaka BM, Gershengorn MC (2012). Taltirelin is a superagonist at the human thyrotropin-releasing hormone receptor. Front Endocrinol (Lausanne) 3: 120.

Thomas TP *et al.* (2008). Dendrimer-epidermal growth factor conjugate displays superagonist activity. Biomacromolecules 9: 603–609.

Thompson AJ, Lummis SC (2013). A single channel mutation alters agonist efficacy at 5-HT3A and 5-HT3AB receptors. B J Pharmacol 170: 391–402.

Valmori D *et al.* (1998). Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. J Immunol 160: 1750–1758.

Wess J, Liu J, Blin N, Yun J, Lerche C, Kostenis E (1997). Structural basis of receptor/G protein coupling selectivity studied with muscarinic receptors as model systems. Life Sci 60: 1007–1014.